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Molecular diversity and transferability of the tetracycline resistance gene *tet*(M), carried on Tn*916-1545* family transposons, in enterococci from a total food chain

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Abstract In the present study, 20 enterococci belonging to the species *Enterococcus faecalis* (12 strains), *Enterococcus faecium* (4), *Enterococcus durans* (2), *Enterococcus hirae* (1) and *Enterococcus mundtii* (1) and originating from a total production chain of swine meat commodities were analysed to investigate the diversity of their tetracycline resistance gene *tet*(M). PCR–RFLP and sequence analysis showed that the *tet*(M) gene of most strains can be correlated with the Tn*916* transposon. Conversely, *tet*(M) of six *E. faecalis* and the *E. hirae* strain, all isolated from pig faecal samples, may be associated with previously undescribed members of the Tn*916-1545* transposon family. In vitro filter conjugation trials showed the ability of 50% of the enterococcal strains, including *E. mundtii*, to transfer the *tet*(M) gene (and the associated Tn*916* and new transposons) to *E. faecalis* or *Listeria innocua* recipient strains. *tet*(M) gene transfer to *L. innocua* recipient was also directly observed in meat food products. Collectively, these sequence and conjugation data indicate that various transposons can be responsible of the spread of tetracycline resistance in enterococci and validate the opinion that *Enterococcus* species are important sources of antibiotic resistance genes for potentially pathogenic bacteria occurring in the food chain.

Keywords Conjugation · Enterococci · *Listeria* · *tet*(M) · Tetracycline resistance · Tn*916*

Introduction

Enterococcus spp. are ubiquitous bacteria that are very common in a number of the different environments comprising the gastrointestinal tract of mammals and other animals, as well as food such as meat, milk and cheese. On the other hand, they are considered as emerging pathogens of humans and are often identified as the cause of an increasing number of hospital-acquired infections. One of the reasons for the rise of infections related to enterococci might be their ability to develop resistance against a wide variety of antibiotics. In fact, enterococci are well-known reservoirs and vehicles of antibiotic resistance and a

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number of studies have dealt with the distribution of antibiotic resistance genes in strains isolated from enteric habitats and, more recently, from food products and samples collected in various steps of total food chains (Aarestrup et al. 2000; Huys et al. 2004; Rizzotti et al. 2005; Wilcks et al. 2005; Hummel et al. 2007).

Resistance to tetracycline is a highly prevalent phenotypic trait among enterococci and several classes of tetracycline resistance genes have been identified in these bacteria. Different mechanisms are implicated in resistance against tetracycline, such as ribosome protection, encoded for example by *tet*(M), *tet*(O) and *tet*(S) genes, and efflux systems, encoded by *tet*(K) and *tet*(L). The widespread distribution of *tet*(M) in many bacterial genera, including *Enterococcus* (Clewell et al. 1995; Roberts 1996), is often linked to the presence of conjugative transposons of the Tn*916-1545* family. Mating experiments have demonstrated the ability of some *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* strains to transfer tetracycline resistance determinants by means of Tn*916-1545* transposons (Huys et al. 2004; Wilcks et al. 2005; Hummel et al. 2007). However, the presence and distribution of different members of this transposon family in the enterococcal population of the food environment as well as their transfer to other food-borne bacteria has not been extensively investigated.

It is known that the *tet*(M) gene found in different bacteria shows several mosaic structures, probably as a result of homologous recombinations (Oggioni et al. 1996; Doherty et al. 2000). Investigations on the genetic structure of the resistance determinants may help to obtain insight into their evolution and distribution among enterococci and consequently to recognise the transposons most frequently involved in tetracycline resistance gene transfer.

The aim of the present study was to analyse the diversity of the tetracycline resistance gene *tet*(M) in taxonomically well-characterised enterococcal strains isolated from a total production chain of swine meat commodities using a combination of PCR–restriction fragment length polymorphism (PCR–RFLP) and DNA sequencing. Additionally, mating experiments were conducted in vitro and in food matrices to assess the transferability of various *tet*(M)-carrying transposons of the examined strains to other food bacteria.

Materials and methods

Bacterial strains, cultural methods and susceptibility testing

The 20 tetracycline-resistant *Enterococcus* strains used in this study are listed in Table 1. These enterococci were originally isolated from samples of the total production chain of swine meat commodities, identified and genotypically characterised in a previous study (Rizzotti et al. 2005).

E. faecalis OG1RF (resistant to rifampicin and fusidic acid) and *L. innocua* LMG 11387^T, both susceptible to tetracycline, were used as recipient strains in mating experiments.

All strains were grown in Brain Heart Infusion (BHI) medium (Fluka, Milan, Italy) at 37°C for 24 h, unless otherwise indicated.

Minimum Inhibitory Concentrations (MICs) of tetracycline were determined by the broth dilution method according to standards set by CLSI (Clinical and Laboratory Standards Institute, former NCCLS, Wayne, USA) in Iso-sensitest broth (Oxoid Italia, Milan, Italy) supplemented with 10% BHI.

DNA extraction and gene-specific PCR assays

Total genomic DNA was extracted and purified from 2-ml cultures as described by Marmur (1961).

Detection of the tetracycline resistance genes *tet*(M), *tet*(K) and *tet*(O) were performed as previously described (Rizzotti et al. 2005). The genes *tet*(L) and *tet*(S) were amplified with the primers reported by Trzcinski et al. (2000) and Ng et al. (2001), respectively, using 2 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each specific primer and 0.05 U μl^{-1} of *Taq* polymerase. DNA amplification of *tet*(L) was carried out using the following conditions: a 5-min initial denaturation at 94°C followed by 30 cycles of 94°C for 45 s, 61°C for 45 s and 72°C for 45 s. The PCR for *tet*(S) consisted of a 5-min denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. A final extension for 5 min at 72°C was performed for both reactions. Occurrence of the Tn*916-1545* transposon family was determined using the primers designed for the integrase (*int*) gene as described by Doherty et al. (2000). In addition, the primers tetM-13985 (5′-CCGTCGTCCAAATAGTCGGA-3′) and traA-16221 (5′-ATACTCATTGCCTGCGACGG-3′) were newly

Table 1 Enterococcal strains used in this study

Species	Strain	Source of isolation	Tetracycline resistance genes[a]	Tetracycline MIC (μg/ml)	PCR–RFLP profile type[b]
E. durans	ET24	Dry sausage	*tet*(M), *tet*(K), *tet*(L)	256	1
	ET27	Dry sausage	*tet*(M), *tet*(K), *tet*(L)	256	1
E. faecalis	EE3	Pig faeces	*tet*(M), *tet*(L)	256	4
	EE10	Pig carcass	*tet*(M), *tet*(K), *tet*(L)	256	1
	EE17	Raw minced pork	*tet*(M), *tet*(L)	256	1
	EE32	Pig faeces	*tet*(M), *tet*(L)	128	3
	EG21	Pig faeces	*tet*(M), *tet*(K)	128	3
	ET33	Pig faeces	*tet*(M)	128	3
	ET35	Pig faeces	*tet*(M)	128	3
	ET42	Pig carcass	*tet*(M), *tet*(O)	256	1
	ET47	Raw minced pork	*tet*(M), *tet*(K), *tet*(O), *tet*(L)	128	1
	ET48	Pig faeces	*tet*(M), *tet*(K), *tet*(L)	128	3
	ET55	Pig carcass	*tet*(M)	256	1
	ET57	Raw minced pork	*tet*(M), *tet*(K)	128	1
E. faecium	EO5	Pig faeces	*tet*(M), *tet*(K)	128	1
	ET6	Feedstuff	*tet*(M)	64	1
	ET17	Pig carcass	*tet*(M), *tet*(K)	256	1
	ET52	Feedstuff	*tet*(M), *tet*(K)	128	2
E. hirae	ET36	Pig faeces	*tet*(M), *tet*(K), *tet*(O), *tet*(L)	>256	4
E. mundtii	ET39	Feedstuff	*tet*(M), *tet*(K), *tet*(O), *tet*(L)	256	1

[a] No strain carried the *tet*(S) gene

[b] Profiles obtained by PCR–RFLP of the 1,777 bp *tet*(M) gene fragment

designed based on the Tn*916* sequence to amplify a 2,256-bp fragment comprising the *tet*(M)-*traA* region of this transposon and similar ones belonging to the Tn*916-1545* family. The reaction was conducted with 1.5 mM $MgCl_2$, 300 μM dNTPs, 0.6 μM of each primer and 0.05 U μl^{-1} of *Taq* polymerase. After an initial denaturation of 4 min at 94°C, 25 cycles of 1 min at 94°C, 1 min at 51°C, and 2 min at 72°C and a final extension for 6 min at 72°C were performed. Resistant enterococcal strains were used as positive controls in all the specific amplification reactions. To amplify a 1,777-bp fragment of the *tet*(M) gene, the primer *tet*(M)-reverse (Doherty et al. 2000) coupled with tetM-1 (Rizzotti et al. 2005) were used.

PCR–RFLP and sequencing of the *tet*(M) gene

In silico restriction analysis of the 1,777-bp fragment of the *tet*(M) gene was performed using the on-line available program NEBcutter V2.0.

For the PCR–RFLP analysis, the *tet*(M) fragment was digested with the endonucleases *Hinf*I (Promega, Milan, Italy), *Taq*I (Roche, Milan, Italy) and *Hpa*II (Roche), using 5 U of enzyme in a reaction volume of 20 μl. The fragments obtained were separated on a 2% agarose gel.

For sequencing, the PCR products were purified with the Wizard SV Gel and PCR Clean-Up system according to the manufacturer's instructions (Promega Corporation, Madison, Wis.) and sent to BMR-Genomics (Padova, Italy). The BlastN program was used for sequence similarity searches.

Phylogenetic trees were calculated with parsimony and distance analysis, with maximum likelihood distance estimation and neighbour joining tree reconstruction, as implemented in MEGA4 (Tamura et al. 2007). In these analyses the sequence length was reduced to cover a region that is present in all the chosen sequences.

Reference sequences used in PCR–RFLP and phylogenetic analysis

The following *tet*(M) sequences carried by different transposons of the Tn*916-1545* family were used in PCR–RFLP and phylogenetic analysis: accession number U09422 (*E. faecalis* DS16, transposon Tn*916*), AM411377 (*Streptococcus pyogenes* A-3, Tn*1116*), X04388 (*E. faecalis*, Tn*1545*), AF376746 (*Streptococcus pneumoniae*, Tn*2009*), X90939 (*S. pneumoniae*, Tn*5251*), AF333235 (*Clostridium difficile*, Tn*5397*), AY898750 (*Streptococcus cristatus*, Tn*6002*) and AM410044 (*S. pneumoniae* Ar-4, Tn*6003*). Other *tet*(M) sequences included in the phylogenetic analysis were: AJ585080 (*E. faecalis*), AJ585083 (*E. faecalis*), AY149597 (*Lactobacillus plantarum* isolate DG533, plasmid), DQ223244 (*E. faecium* 9830409-1, plasmid), EU182585 (*Streptococcus suis* T2S3), EU350140 (*S. suis* 3–1) and M21136 (*Staphylococcus aureus*).

Mating experiments and selection of transconjugants

Filter mating experiments were carried out as described by Huys et al. (2004). Mating trials in food matrices were performed by inoculating the surface of fresh pork meat and dry fermented sausage slice samples with equal volumes of donor and recipient cultures. After incubation at 30°C for 2 days and at 10°C for 15 days, the food samples were washed with sterile peptone physiological solution (1 g l^{-1} peptone, 0.85 g l^{-1} NaCl) and the counts of donors, recipients and tranconjugants were determined. BHI medium supplemented with 16 μg ml^{-1} tetracycline or 50 μg ml^{-1} rifampicin plus 25 μg ml^{-1} fusidic acid was used for growth and selective counts of the enterococcal donors and the recipient strain *E. faecalis* OG1RF, respectively. Palcam agar base plus Palcam selective supplement (PALCAM; Oxoid Italia, Milan, Italy) without antibiotics was used for the count of the recipient strain *L. innocua* LMG 11387^T. Transconjugants of *E. faecalis* and *L. innocua* were selected on BHI agar supplemented with tetracycline, rifampicin and fusidic acid at the same concentrations reported above or on PALCAM supplemented with 10 μg ml^{-1} tetracycline, respectively. Presumptive transconjugants were typed to distinguish them from mutants with primers M13 (5′-GAGGGTGGCGGTTCT-3′) or M14 (5′-GAGGGTGGGGCCGTT-3′) using the random amplification of the polymorphic DNA (RAPD) procedures of Zapparoli et al. (2000). They were also subjected to PCR amplifications to detect the presence of *tet*(M) and Tn*916-1545* transposons sequences as indicated above.

Nucleotide sequence accession numbers

The nucleotide sequences of the *tet*(M) gene fragments from the strains ET42, ET52, EE3 and ET35 were deposited in the GenBank database under accession nos. FM202720, FM202721, FM202722 and FM202723, respectively.

Results

Detection of tetracycline resistance genes and Tn*916-1545* transposons in the analysed enterococci

The tetracycline-resistant enterococci analysed in this study have been isolated from several samples obtained from various steps of the total production chain of swine meat commodities (Rizzotti et al. 2005). The isolates have been identified as *E. faecalis* (12 strains), *E. faecium* (4), *E. durans* (2), *Enterococcus hirae* (1) and *Enterococcus mundtii* (1) and proved to be different at the genetic level. As shown in Table 1, all strains carried the gene *tet*(M), 12 hold *tet*(K) and four carried *tet*(O) (Rizzotti et al. 2005).

In the present study, the strains were investigated for the occurrence of other two common tetracycline resistance genes, *tet*(L) and *tet*(S). The gene *tet*(L) was observed in the 50% of the strains, whereas *tet*(S) was not detected (Table 1). In addition, all strains possessed a transposon of the Tn*916-1545* family, as they carry the gene *int* coding for the integrase typical of these mobile elements (Clewell et al. 1995). The association of the *tet*(M) gene with this type of transposon was confirmed by PCR as a *tet*(M)-*traA* fragment band was obtained from all strains (data not shown).

Tetracycline resistance levels of the analysed enterococci

MIC values of tetracycline were determined for the 20 strains carrying different combinations of tetracycline

resistance genes. MIC levels ranged from 64 to >256 μg ml^{-1}. The MIC of each isolate was compared with the occurrence of tetracycline resistance determinants. No correlation was found between the MIC values and the presence of one or more resistance genes as well as different combinations of these genes or the presence of genes that code for different resistance mechanisms (ribosome protection or protein efflux).

PCR–RFLP of *tet*(M) gene in the analysed enterococci

To obtain insights into the diversity of the *tet*(M) gene carried by different transposons of the Tn*916-1545* family, a preliminary in silico restriction analysis was conducted on eight reference *tet*(M) sequences retrieved from Genbank and representing the transposons listed in the Materials and methods section. This analysis showed that the digestion with the selected endonucleases *Hinf*I, *Taq*I and *Hpa*II generated restriction patterns of the *tet*(M) gene suitable for the distinction of the different transposons, except for Tn*2009*, Tn*6002* and Tn*5251* (see Supplementary Table S1, available online).

Experimental application of these findings by a PCR–RFLP analysis of a 1,777-bp fragment of the *tet*(M) gene provided information on the likely type of conjugative transposon carried by the strains analysed. Different profiles (termed monoprofiles) were produced by using each of the three above-mentioned endonucleases: monoprofiles A, A′ and α were obtained with the enzyme *Hinf*I on the 20 studied strains; B, B′ and β with the enzyme *Taq*I; C and C′ with *Hpa*II (Fig. 1). The observation of the monoprofiles obtained with the three enzymes allowed the strains to be divided into four groups with different combinations of restriction profiles: A-B-C (profile 1), A-β-C (profile 2), A′-B′-C′ (profile 3) and α-B′-C′ (profile 4). Thus, four different classes of PCR–RFLP profiles (1–4) were generated from the 20 enterococcal strains (Table 1) and they differed in the monoprofiles obtained by one, two or three enzymes. The PCR–RFLP profile 1, the most frequent (12 out of 20) in the enterococci of this study, corresponded to those expected for the *tet*(M) allele carried by transposon Tn*916*. On the contrary, the other profiles obtained did not match any of those expected for the *tet*(M) gene of the reference transposons listed above.

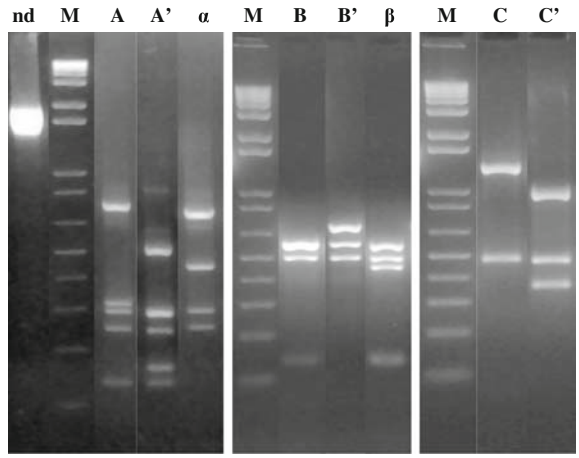

Fig. 1 PCR–RFLP analysis of the 1,777-bp fragment of *tet*(M) gene of different enterococcal strains. Examples of the monoprofiles obtained using the indicated endonuclease enzymes are reported. *Lane M* 1 kb Plus DNA Ladder molecular weight marker (Invitrogen); nd, not digested fragment

Sequence analysis of a *tet*(M) gene fragment of representative enterococci

A *tet*(M) gene fragment of the enterococcal strains ET42, ET52, ET35 and EE3, chosen as representative of each PCR–RFLP profile, was sequenced. The obtained sequences (more that 85% of the total gene length) were subjected to BLAST analysis in the GenBank database.

The dataset comprising the sequences of the *tet*(M) gene of the Tn*916-1545* transposons selected as references, those derived from the public database (i.e. two related *S. suis* sequences) and the sequences newly obtained in this study was used to calculate the degree of genetic diversity (Table 2). The number of different nucleotides varied from zero to 155, which corresponds to a divergence of up to 10.9% of the *tet*(M) sequence under consideration.

For the construction of a phylogenetic tree, the sequences representative of the *tet*(M) sequence homology groups (SHGs) described by Gevers et al. (2003) and Huys et al. (2004) were also considered. In accordance with these authors, SHGs were delineated on the basis of an internal sequence identity level higher or equal to 99.6%. In the tree (Fig. 2), the *tet*(M) sequences fell into three major groups. The sequence of the *tet*(M) gene fragments of ET42 and

Table 2 Number of differences among the 1,422-bp *tet*(M) sequences of four selected enterococcal strains and transposons chosen as references; the *tet*(M) sequences of the *S. suis* strains T2S3 and 3–1 (acc. no. EU182585 and EU350140, respectively) were also included

tet(M) sequences	No. of differences												
	ET35	ET42	ET52	Tn*916*	Tn*1116*	Tn*1545*	Tn*2009*	Tn*5251*	Tn*5397*	Tn*6002*	Tn*6003*	EU182585	EU350140
EE3	63	82	112	88	124	90	79	78	53	88	90	63	1
ET35		103	113	103	87	79	72	71	42	63	79	0	64
ET42			36	12	110	86	61	60	135	72	86	103	83
ET52				32	132	122	97	96	155	104	122	113	113
Tn*916*					112	92	67	66	135	74	92	103	89
Tn*1116*						61	56	55	119	43	63	87	125
Tn*1545*							29	28	85	20	4	79	91
Tn*2009*								1	104	15	31	72	80
Tn*5251*									103	14	30	71	79
Tn*5397*										95	85	42	54
Tn*6002*											20	63	89
Tn*6003*												79	91
EU182585													64

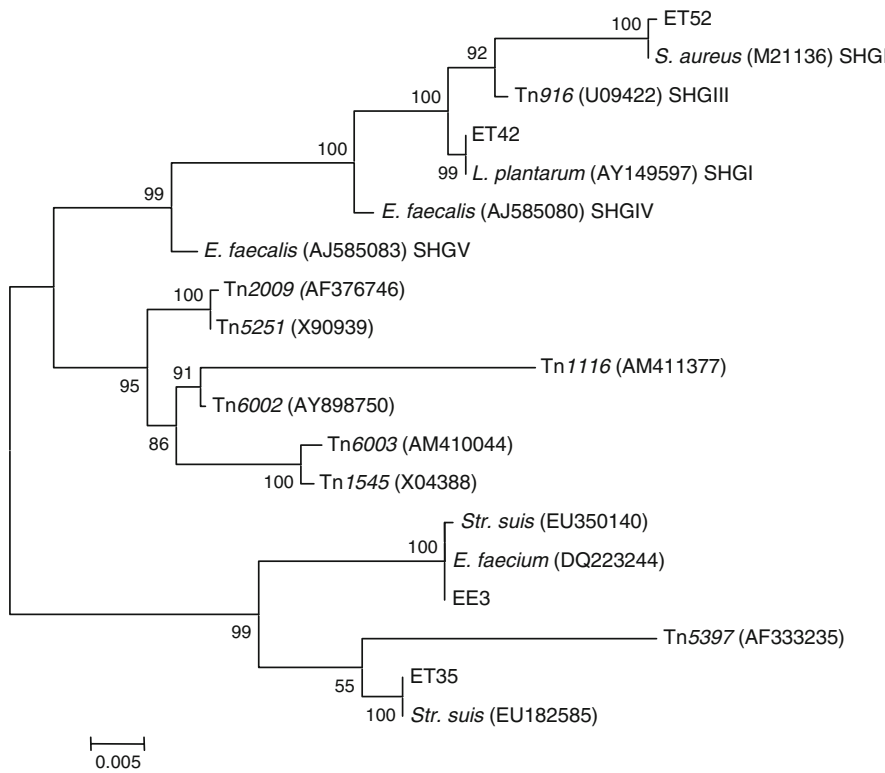

Fig. 2 Phylogenetic tree obtained from the multiple alignment of the four enterococcal *tet*(M) nucleotide sequences obtained in this study and 15 other *tet*(M) gene sequences available in GenBank. Almost identical topologies were obtained with parsimony and distance matrix analysis; the *latter* is shown in the figure. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The *bar* indicates the number of nucleotide substitutions per site. GenBank accession numbers are in *parentheses*

ET52 (PCR–RFLP profile 1 and 2, respectively) showed high similarity with a number of *tet*(M) sequences including that of Tn*916* transposon from *E. faecalis* DS16 (acc. no. U09422). Hence, the first group comprised the sequences of the two strains ET42 and ET52 and those related to the transposon

Tn*916*, including the sequences of the five SHGs (from SHG I to SHG V) of *tet*(M). The ET42 sequence representative of profile 1 was identical (zero nucleotide substitution) to the *tet*(M) sequence of *L. plantarum* (acc. no. AY149597) that represent SHG I. ET52 can be assigned to SHG II as it differed in only two nucleotides (sequence identity >99.6%) from the *S. aureus* sequence (acc. no. M21136) belonging to this SHG. No sequences were assigned to the other *tet*(M) SHGs. A second group of the tree included the strains ET35 and EE3 and the transposon Tn*5397*. ET35 (representative of profile 3) was closely related to a tetracycline resistance gene associated with a strain of *S. suis* isolated from pigs (acc. no. EU182585). EE3 (representative of profile 4) was almost identical to an enterococcal plasmid-located *tet*(M) sequence (acc. no. DQ223244) and to a sequence derived from another *S. suis* strain (acc. no. EU350140). Finally, the third group of the tree clustered all the other reference transposons.

Tetracycline resistance transfer experiments in vitro and in food matrices

To investigate the transferability of the *tet*(M) gene, filter mating trials were conducted in vitro using the 20 enterococci as donors and *E. faecalis* OG1RF and *L. innocua* LMG 11387^T as recipient strains. A representative number (5–10) of presumptive transconjugants, grown onto plates containing the selective agent tetracycline (plus rifampicin and fusidic acid for the recipient OG1RF), was isolated from each mating experiment and subjected to RAPD analysis to exclude the presence of mutant donors (data not shown).

Five out of 20 donors (four *E. faecalis* and one *E. faecium* strain) were able to transfer tetracycline resistance to the recipient *E. faecalis* strain (Table 3). None of the strains belonging to the other species transferred tetracycline resistance to this recipient. Unexpectedly, a higher number of strains (eight) conjugated with the recipient *L. innocua* strain, i.e. five strains of *E. faecalis*, two of *E. faecium* and the *E. mundtii* strain. Transfer frequencies ranged from 10^{-5} to 10^{-8} transconjugants per recipient (Table 3).

Three donors, ET17, ET42 and ET55, which transferred tetracycline resistance to both recipient strains in filter mating experiments, were used for mating trials in food matrices. As the sources of isolation of the donors are meat samples, this type of products was chosen for the experiment. One of the three donors, *E. faecalis* ET42, conjugated with *L. innocua* in both meat products at a temperature of 30°C. Conversely, mating trails conducted at 10°C did not produce transconjugants.

Table 3 Results of the mating experiments for the 10 enterococcal strains that gave a positive outcome

Mating trial	Donor[a]	Recipient[b]	Transfer frequency (Tc/rc)[c]
On filter			
	EE10	*Efs*	2.0×10^{-7}
	EE17	*Efs*	1.6×10^{-6}
	ET6	*List*	3.5×10^{-7}
	ET17	*Efs*	2.0×10^{-8}
		List	8.0×10^{-8}
	ET35	*List*	2.5×10^{-7}
	ET39	*List*	8.0×10^{-8}
	ET42	*Efs*	6.0×10^{-7}
		List	1.3×10^{-5}
	ET47	*List*	5.0×10^{-6}
	ET55	*Efs*	2.4×10^{-7}
		List	8.0×10^{-7}
	ET57	*List*	2.0×10^{-6}
In food matrix			
	ET42	*List*	(M) 1.8×10^{-6}
		List	(S) 8.3×10^{-8}

[a] All strains have PCR–RFLP profile 1 except ET35 that shows profile 3

[b] *Efs*: *E. faecalis* OG1RF; *List*: *L. innocua* LMG 11387

[c] Tc/rc, transconjugants/recipient; (M) and (S), mating experiments conducted on slices of fresh pork meat and dry fermented sausage, respectively

Characterization of transconjugants

tet(M)-specific PCR was carried out to verify the transfer of the *tet*(M) gene to transconjugants, which were selected during the experiments solely by their tetracycline resistance phenotype. Results revealed that all the isolates had acquired the *tet*(M) gene. Moreover, the *int* gene specific for the Tn*916-1545* transposons was found in all transconjugants, together with positive PCR results for the amplification of the *tet*(M)-*traA* region of the transposon.

Phenotypic tests showed the transconjugants displayed a tetracycline MIC of 128 or 256 μg ml^{-1} for

both enterococcal and *Listeria* isolates, which is at the same level or lower than those of the corresponding donor.

Discussion

The genes encoding for tetracycline resistance are numerous but the most widely distributed tetracycline resistance determinant in Gram-positive bacteria is the *tet*(M) gene (Roberts 1996). Previous studies have shown the diversity of the *tet*(M) gene in different bacterial strains and species (Huys et al. 2004; Bertrand et al. 2005; Spigaglia et al. 2006). The in silico analysis on reference *tet*(M) sequences conducted in this study confirmed that several nucleotide differences are present in the tetracycline resistance gene of different members of the Tn*916-1545* transposon family (Table 2; Online Supplementary Table S1).

The PCR–RFLP method here employed allowed the differentiation of the *tet*(M) sequences of the analysed strains and yielded information on their degree of nucleotide polymorphism. This is the first application of this method to link *tet*(M) gene variability with the type of transposon present. The most frequently found PCR–RFLP profile (profile 1) can be correlated to the sequence of the *tet*(M) gene carried by a Tn*916* transposon. Interestingly, this profile type was mainly linked with strains belonging to different enterococcal species isolated from the last steps of the food production chain such as pig carcasses, raw minced pork or sausages. The *tet*(M) sequence that gave profile 2 (as represented by *E. faecium* ET52) can be associated with the Tn*916* transposon, too, although it demonstrates minor sequence divergence (32/1,422 different nucleotides, 2.2%).

The profile 3, derived from five *E. faecalis* strains (as represented by ET35), was the second most frequent one in this study and was found only in pig faecal isolates. This isolation source may explain the similarity with a *tet*(M) sequence from a *S. suis* isolate. Similar considerations may also apply for *E. faecalis* EE3 and *E. hirae* ET36 which gave RFLP profile 4. Regarding the transposons carrying these *tet*(M) sequences, as the *tet*(M) sequences obtained were very divergent from those carried by the available reference transposons, we can hypothesise that they may be associated with as yet undescribed members of the Tn*916-1545* family. The definition of the type of transposon present in these isolates requires further investigation.

Phylogenetic trees showing the relationships among the *tet*(M) genes carried by different bacteria or mobile elements were obtained in recent studies (Huys et al. 2004; Bertrand et al. 2005; Agersø et al. 2006). The tree constructed in this study comprised a total of 19 *tet*(M) sequences, four new sequences from our enterococcal strains and 15 retrieved from Genbank. The tree (Fig. 2) showed three major groups, and the *tet*(M) gene of the four newly obtained enterococcal sequences fell in two of these. The strains ET42 and ET52 (PCR–RFLP profiles 1 and 2, respectively) grouped with the Tn*916 tet*(M) sequence, thus confirming their correlation with the Tn*916* transposon. This result is also validated by the high bootstrap values of the branch. However, the two sequences belong to different SHGs, as defined by Huys et al. (2004).

The *tet*(M) of transposon Tn*5397* was included in the group comprising the pig faecal isolates EE3 and ET35, the two *S. suis* sequences and an *E. faecium* plasmid-located sequence. The Tn*5397*-derived sequence is quite distant from the others (40–50 different nucleotides) and, in addition, this transposon is characterised by the absence of the *int* gene, that was detected in both *E. faecalis* strains EE3 and ET35. A similar situation was observed in the *tet*(M) tree obtained by Agersø et al. (2006), who found a group clustering Tn*5397* with plasmid-located *tet*(M) sequences not associated with this type of transposons. Hence, despite the linkage with Tn*5397*, strains EE3 and ET35 are likely associated with different (as yet undescribed) transposons of the Tn*916-1545* family that needs further study.

As previously observed by Huys et al. (2004), the *tet*(M) gene is frequently transferred in mating experiments between various species of enterococci. In the present study, this finding was confirmed and the capability of *E. mundtii* to act as a donor of this tetracycline resistance gene was also demonstrated. This ability can be explained by the presence of a Tn*916-1545* transposon in all tested donors. Interestingly, nine out of the 10 enterococci that were able to conjugate hold a *tet*(M) gene associated to a Tn*916* transposon; thus, we can hypothesise that these transposons possess a higher capability to be transferred between a bacterial cell to another.

Fifty percent (10/20) of the analysed donors were able to transfer tetracycline resistance to one of the recipients: 25% to *E. faecalis* and 40% to *L. innocua*. The values obtained in the matings towards the enterococcal recipient are similar to those reported in literature, which vary from 2.6 to 31% of the donors (Huys et al. 2004; Wilcks et al. 2005; Hummel et al. 2007). Unexpectedly, mating towards *Listeria* took place at a higher rate and our results showed that such transfer events could easily occur. To our knowledge, the only previous study describing the transfer of a tetracycline resistance gene from enterococci to *Listeria* spp. was conducted with a strain carrying a Tn*1545* transposon (Doucet-Populaire et al. 1991). Conjugal transfer of resistance determinants was previously observed in food matrices between donors and recipient enterococcal strains (Cocconcelli et al. 2003). Our results demonstrated that this transfer is also possible between enterococci and *Listeria* at frequencies from 10^{-6} to 10^{-8} transconjugants/recipient.

In conclusion, the results of the current study highlighted the diversity in the *tet*(M) gene sequence in enterococci isolated from a total food chain and thus the likely presence of different transposons driving the spread of tetracycline resistance. Moreover, the data obtained support the conclusion that *Enterococcus* species can be important sources of antibiotic resistance genes for *Listeria* through the transfer of mobile genetic elements, such as transposons. Since these bacteria can be frequently found in the same habitats, a horizontal spread of resistance to *Listeria* spp. could be possible in some steps of the food production chain.

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